Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis

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Abstract

Electroporation of cells with short, high-voltage pulses causes a transient permeabilisation of cell membranes that permits passage of otherwise non-permeating ions and molecules. In this study, we illustrate how electroporation with isotonic calcium can achieve highly effective cancer cell kill in vivo. Calcium electroporation elicited dramatic antitumor responses in which 89% of treated tumors were eliminated. Histological analyses indicated complete tumor necrosis. Mechanistically, calcium electroporation caused acute ATP depletion likely due to a combination of increased cellular use of ATP, decreased production of ATP due to effects on the mitochondria, as well as loss of ATP through the permeabilised cell membrane. Taken together, our findings offer a preclinical proof of concept for the use of electroporation to load cancer cells with calcium as an efficient anti-cancer treatment. Electroporation equipment is already used clinically to enhance the delivery of chemotherapy to superficial tumors, with trials on internal tumors in progress, enabling the introduction of calcium electroporation to clinical use. Moreover, the safety profile, availability, and low cost of calcium facilitate access to this technology for many cancer patients in developed and developing countries.

Introduction

Calcium is a ubiquitous second messenger involved in many cellular processes, including regulation of transcription, metabolism, proliferation, muscle contraction, and cell death (apoptosis and necrosis) (1,2,3). Due to the many effects of calcium, the intracellular calcium concentration is tightly regulated, and the effects of calcium are dependent on time, place, amplitude, frequency, and duration of the calcium signal (1,2,4,5,6). Cellular uptake of calcium can be facilitated by electroporation, where cells are exposed to an electric field exceeding the dielectric strength of the cell membrane, resulting in generation of reversible permeabilisation structures in the membrane.
Calcium loading by electroporation causes tumor necrosis (7). In eukaryotic cells the concentration of free intracellular calcium is very low (10^{-7}M), in striking contrast to the concentration of free calcium in plasma (10^{-3}M) (5). Thus, even a small increase in the permeability of the membrane may increase the concentration of free intracellular calcium drastically. Increase in intracellular calcium concentration due to electroporation has previously been shown (8,9) but its use in cancer treatment was not investigated.

Here we document that calcium electroporation can be highly efficient in eradicating tumors in vivo, and we suggest that the mechanistic explanation is acute energy depletion.

Materials and Methods

In vitro electroporation

Three cell lines were used for in vitro experiments, DC-3F, a transformed Chinese hamster lung fibroblast cell line; K-562, a human leukemia cell line; and Lewis Lung Carcinoma, a murine lung carcinoma cell line (all tested negative for mycoplasma). Cells grew in RPMI 1640 culture medium (Gibco, Invitrogen), 10% fetal calf serum (Gibco, Invitrogen), penicillin, and streptomycin at 37°C and 5% CO₂. After harvesting, cells were washed and diluted in HEPES buffer (10mM HEPES (Lonza), 250mM sucrose and 1mM MgCl₂ in sterile water). 270µl cell suspension (6.1 x 10^6 cells/ml) with 30µl CaCl₂ or HEPES buffer (controls) were electroporated in 4mm cuvettes with aluminium electrodes (Molecular BioProducts, Inc.). Cooled cells (8°C) were exposed to 8 pulses of 99µs with respectively 1.2kV/cm (DC-3F and K562) and 1.4kV/cm (Lewis Lung Carcinoma) using a BTX T820 square wave electroporator. Electroporation parameters were optimized for high permabilisation and cell survival. After 20 min at 37°C and 5% CO₂ cells were diluted in culture medium (as above) and seeded in 96-well plates (3.1x10^6 cells/100µl). After respectively 1 and 2 days, MTT assay was performed with Multiskan-Ascent ELISA reader (Thermo Labsystems).
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Difference between electroporated and non-electroporated cells in identical buffer was assessed using two-way analysis of variance (ANOVA) with post least-squares-means test with Bonferroni correction.

**ATP assay**

DC-3F cells were electroporated as described above, with 1mM calcium. Cells electroporated with HEPES buffer, non-electroporated cells with 1mM calcium, and untreated cells were used as controls. Cell death induced by irreversible electroporation (8 pulses of 99µs and 6.6kV/cm) was used as negative control. Cells seeded in 96-well plates as above were lysed using Cell-Based Assay Lysis Buffer (Cayman chemical) and ATP content was determined after 1, 4 and 8 hours incubation by adding 100µl rL/L Reagent (ENLITEN ATP assay, Promega) and measuring light emission using a luminometer (LUMIstar, Ramcon).

Difference in ATP level after different treatments was assessed using two-way ANOVA with post least-squares-means test with Bonferroni correction.

**Tumor volume and tumor fluorescence intensity**

*In vivo* experiments were performed in accordance with European Convention for the Protection of Vertebrate Animals used for Experimentation and with approval from the Danish Animal Experiments Inspectorate.

For *in vivo* experiments the H69 human small cell lung cancer cell line stably transfected with EGFP regulated by the cytomegalovirus (CMV) promoter (10) was kindly provided from Department of Radiation Biology, Copenhagen University Hospital. Cells were tested by rapid MAP27 panel (Taconic) without signs of infection. Cells grew *in vitro* as described above. 1.5x10^6 cells/100µl PBS were injected subcutaneously in both flanks of 9-11 weeks old NMRI-Foxn1nu
Calcium loading by electroporation causes tumor necrosis in mice (Harlan). Tumor pieces were transplanted to the right flank of recipient mice. Hypnorm-dormicum (VetaPharma and Roche) was used for anesthesia complemented with rimadyl (Pfizer Aps) and lidocaine in the incision. Mice were randomised at an average tumor diameter of 6.2mm (range 5.5-6.9 mm) and treated with 1) injection of isotonic calcium-chloride solution (168mM CaCl\(_2\)) and electroporation (8 pulses of 100µs at 1.0 kV/cm and 1 Hz) (7,11) using a 6mm plate electrode and a square wave electroporator (Cliniporator, IGEA), 2) calcium-free physiological saline injection and electroporation (same parameters as above), 3) isotonic calcium-chloride injection, or 4) calcium-free physiological saline injection. Atomic absorption spectrophotometry (Solaar AAS, Thermo) confirmed absence of calcium in physiological saline. Tumor volume was calculated as \(ab^2\pi/6\) (\(a\) largest diameter, \(b\) largest diameter perpendicular to \(a\)). Initially, tumors were injected with a volume equivalent to the tumor volume but as the Ca\(^{2+}\)-EP group showed skin necrosis, the injected volume was changed to half the tumor volume for all groups. Solutions were injected through the side of the firm tumor and the needle was moved around inside the tumor to secure injection all over the tumor. Tumor size measurements (Vernier calliper), and bioimaging using the Optix MX-2 optical molecular image system (ART) with a scan resolution of 1.5mm, were performed before treatment and three times a week after treatment. Background fluorescence (opposite flank) was subtracted from fluorescence intensity of tumors, and fluorescence intensity below 100 normalised counts (NC) was filtered away using Optiview version 2.02 (ART).

Differences in tumor volume and fluorescence intensity in the 4 treatment groups were evaluated as repeated measurements, validated and analysed with an exponential decrease model with Bonferroni correction using SAS software version 9.1. ‘Group’, ‘days’ and ‘mouse’ were considered factors and baseline levels of tumor volume or fluorescence intensity were used as covariant. Fluorescence intensity values were log transformed before analysis.
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Histology

At an average diameter of 6.1mm (range 5.8-6.6mm) tumors were treated with CaCl₂ injection and electroporation as described above. Tumors were removed respectively 2 hours, 1, 2 and 6 days after treatment, fixated in formalin (10% neutrally buffered) and paraffin embedded. Tissue sections (3μm) were HE-stained, and fraction of necrosis was estimated by stereological point counting using a light microscope, evaluated by a pathologist, blinded with respect to treatment status. Difference in fraction of necrosis was assessed using one-way ANOVA with post least-squares-means test with Bonferroni correction.

Results and Discussion

To test the effect of calcium overloading in vitro we electroporated three cell lines from different species and of different tissue origin in buffers with increasing calcium concentrations (fig. 1). There was a dose dependent, dramatic decrease in viability for all electroporated cell lines with EC₅₀ being 0.57mM Ca²⁺ (range 0.35-0.79mM) whereas EC₅₀ was not reached without electroporation. As expected, due to differences in e.g. cell size and homogeneity there was a differential effect of the electroporation procedure alone on the different cell lines (12), as electroporation alone reduced viability by respectively 0% (DC-3F), 6% (Lewis Lung Carcinoma) and 23% (K-562). Consequently, values are listed as a percentage of electroporated respectively non-electroporated controls. The decrease in viability after calcium electroporation was similar to the effect induced by electroporation with the chemotherapeutic agent bleomycin in concentrations from 0.1µM (data not shown).

After having shown a robust anti-cancer effect in vitro, the effect of calcium electroporation in vivo was tested. Fluorescent tumors (10) were treated with isotonic calcium-chloride injection and electroporated (‘Ca²⁺-EP’) or in the case of controls, injected with calcium-free physiological saline
Calcium loading by electroporation causes tumor necrosis and electroporated (‘NaCl-EP’), only injected with isotonic calcium-chloride (‘Ca\(^{2+}\)’) or calcium-free physiological saline (‘NaCl’) (fig. 2a). Strikingly, Ca\(^{2+}\)-EP treatment eliminated 89% (8/9) of the treated tumors. Ulceration occurred in all Ca\(^{2+}\)-EP treated tumors, with healing at an average of 18 days (range 9-24 days). Tumor volume was measured including the ulceration, giving the impression that tumor volume was increasing just after treatment, however fluorescence intensity showed acute decrease. The volume of tumors treated with Ca\(^{2+}\)-EP was significantly different from controls Ca\(^{2+}\) (p<0.0001) and NaCl-EP (p<0.01). Tumor volume of all non-electroporated tumors increased with a doubling time of respectively 3.9 days (NaCl) and 6.3 days (Ca\(^{2+}\)). Tumors treated with NaCl-EP decreased in size in the first days after treatment but tumors started increasing in size from around day 7, except for two tumors that were eliminated, indicating that electroporation alone could modulate tumor growth.

Optical bioimaging was used *in vivo* to consecutively track the amount of tumor tissue expressing Enhanced Green Fluorescent Protein (EGFP) (10). Fluorescence intensity of Ca\(^{2+}\)-EP treated tumors decreased drastically after treatment and stayed at background levels for the remainder of the experiment, being significantly different from tumors treated with NaCl-EP (p<0.01) and from control groups treated without electroporation (p<0.0001). As expected, fluorescence intensity of the non-electroporated tumors rose over time. The fluorescence intensity of tumors treated with NaCl-EP decreased 2-3 days after treatment and was significantly different from tumors treated with NaCl (p<0.05), thereafter the fluorescence intensity increased and was not significantly different from non-electroporated tumors (fig. 2b-c).

Histological analysis was performed on tissue sections and the fraction of necrosis was estimated. The analysis of Ca\(^{2+}\)-EP treated tumors showed progressive necrosis, which was highly significant 2 days after treatment (p<0.0001) and complete 6 days post treatment (fig. 3a-b).
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Since Ca\textsuperscript{2+}-EP treatment leads to highly efficient cell death independently in different cell lines and also leads to uniform necrosis across tumors within 6 days, a condition \textit{fundamental} for cell survival must be involved. Previous work showed that ATP decreased significantly in tissue exposed to high voltage pulses (13,14). Determination of ATP levels in cells after treatment showed that Ca\textsuperscript{2+}-EP treatment resulted in an immediate and severe drop in ATP level (fig. 3c). Cells treated with electroporation alone exhibited a similar drop in ATP level but with a marked recovery 4 hours after treatment to levels significantly higher than Ca\textsuperscript{2+}-EP treated cells (p<0.0001). Calcium without electroporation did not affect ATP levels. Cells electroporated with calcium-free physiological saline showed a similar drop and recovery in ATP level as cells treated with electroporation alone (data not shown).

Here we show that calcium electroporation leads to acute ATP depletion and cell death (\textit{in vitro}) as well as massive tumor necrosis \textit{in vivo}. As illustrated in fig. 4, ATP depletion in relation to raised intracellular levels of free calcium may be caused by greatly increased activity of the Ca\textsuperscript{2+}-ATPase leading to high consumption of ATP (2,5). Furthermore, a high intracellular calcium level may induce opening of permeability transition pores (PTP) in the mitochondrial membrane, resulting in loss of the electrochemical gradient, the driving force for ATP production, thereby uncoupling mitochondrial formation of new ATP (2,5,6). Other cellular effects associated to calcium overload include activation of lipases and proteases, and generation of reactive oxygen species (ROS), which may also contribute to cell death (2,5,6). Finally, the electroporation procedure itself may lead to some increased ATP consumption as the influx of sodium (directly or due to sodium calcium exchange) may increase Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity (13). A direct loss of ATP through permeabilisation structures is also a possible contributor. All together, this may result in cell death (fig. 4) (2,5,6). Severe calcium overload causes cell death, and depending on the cellular ATP level, cells undergo either apoptosis or necrosis. If the majority of mitochondria remain capable of ATP
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synthesis, the ATP loss may have a transient nature favoring the apoptotic pathway (15,16,17). On the other hand, if ATP depletion is too severe for apoptosis to occur, the cell will undergo necrosis (15,16,17). Our results clearly show that calcium overload induced by calcium electroporation results in severe ATP depletion and necrosis (fig. 3).

Electroporation has been used for local treatment of malignant tumors in combination with chemotherapeutic agents (electrochemotherapy) (11,18) or plasmids (gene electrotransfer) (7) in clinical trials. The cytotoxic agents bleomycin or cisplatin have been used, either by intravenous or intratumoral route, followed by application of electric pulses to the tumor (11,18). Our suggestion would be to use direct intratumoral calcium injection followed by electroporation according to the standard procedures already published for electrochemotherapy (7). We found that injecting isotonic calcium-chloride corresponding to half the tumor volume followed by electroporation was highly efficient. Systemic administration of calcium was not tested in this study as adsorption to plasma proteins and risk of hypercalcemia were predicted. Since electrochemotherapy has proven efficient in cutaneous tumors, electrodes facilitating the application of electroporation for use in colorectal cancer, bone and liver metastases, and brain tumors (19) have been developed, and are now in clinical trials (7). Efforts are also being made to perform irreversible electroporation of tumors (7) where addition of calcium could enhance efficacy. Since calcium for injection is commercially available and regularly used at most hospitals and electric pulses are already used clinically, this treatment could easily be implemented. In addition, calcium has an excellent safety profile both for use in patients and for staff, and would not need administration by staff accredited to administer chemotherapy. Finally, cost of cancer treatment is causing global concern (20), and calcium electroporation is both simple and inexpensive and is likely to be of potential benefit in the treatment of local tumors regardless of histology.
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Reference List


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Figure Legends

**Figure 1. Calcium overloading induces cell death *in vitro***

Cell viability in three cell lines, DC-3F, a transformed Chinese hamster lung fibroblast cell line (■), K-562, a human leukemia cell line (▲), and Lewis Lung Carcinoma (LLC), a murine lung carcinoma cell line (●) after treatment with increasing calcium concentrations either electroporated (red) or not (blue). MTT viability assay was performed respectively 1 (A) and 2 days (B) after treatment. Results are depicted as percentages of controls (electroporated or non-electroporated cells in 0mM calcium) (means±s.d., n≥6). The viability decreased significantly (p<0.01) starting from 0.5mM for all cell lines treated. EP, electroporation.

**Figure 2. Calcium overloading induces cell death *in vivo***

H69 (EGFP transfected human small cell lung cancer cell line) tumors induced on nude mice were treated with calcium-chloride and electroporation (red), calcium-free physiological saline and electroporation (grey), calcium-chloride alone (blue), or calcium-free physiological saline alone (black). Tumor size (A) and fluorescence intensity in bioimager (B) were measured before treatment and 3 times a week after treatment, (means+s.d., n=3-9). C, Representative images of fluorescence intensity in the tumors, placement of the mouse in the scanner and location of the tumor is shown in the top right corner, intensity bar is shown as a logarithmic scale. EP, electroporation; NC, normalised counts.

**Figure 3. Calcium overloading induces tumor necrosis and ATP depletion***

Panel A shows the fraction of necrosis in tumors after calcium electroporation determined by stereological point counting, (median, individual data points (▲), n=4 for treated tumors and n=2
Calcium loading by electroporation causes tumor necrosis for untreated tumors. B, Light microscope images of HE-sections of tumors 2 hours, respectively 6 days after calcium electroporation. C, ATP level in DC-3F cells 1h (dark grey), 4h (grey) and 8h (light grey) after treatment with calcium electroporation, calcium alone or electroporation alone, control (untreated cells), negative control (dead cells). ATP level in percent of control (means+s.d., n=6). Calcium electroporation resulted in an immediate and severe drop in ATP level which stayed low, at 10.3% (p<0.0001) of control levels up to 8 hours after treatment. EP, electroporation.

**Figure 4. Schematic view of the effect of calcium overloading**

Electroporation (EP) generates reversible pores in the cell membrane (1) allowing influx of calcium and sodium, and efflux of potassium and possible also of ATP. Changes in the intracellular ion concentrations lead to high ATP consumption by Ca\(^{2+}\)-ATPases (in the plasma and endoplasmic reticulum membranes) and Na\(^+\)/K\(^+\)-ATPases (2). Calcium overload may induce permeability transition pore (PTP) opening in the mitochondrial membrane resulting in loss of ATP production due to loss of the electrochemical gradient (3), and activation of lipases and proteases, and generation of reactive oxygen species (ROS) (4). This results in severe ATP depletion and necrosis of the cell.
1 Membrane permeabilisation

2 Increased ATP consumption

3 Loss of ATP production

4 Other cellular effects

Lipase and protease activity

\[ \text{Necrosis} \]
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